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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Current therapies in ovarian cancer are limited by the emergence of therapy-resistant cancer cells, also called cancer stem cells, which are often followed by metastatic cancer. As a result, metastatic ovarian cancer remains an incurable disease by current treatment strategies. In our proposal, entitled "*Ovarian Cancer, Stem Cells and Bioreactors*" we have looked for cancer stem cells presence in late stage ovarian cancers, when malignant ascites are building up in patients, a recognized poor prognostic indicator of survival. Our results show that an heterogeneous population of tumor cells is present in patients ascites. No clear phenotypes from patients to patients cancer stem cells could be identified, although the presence of CD133 positive tumor initiating cells were found in at least one of the four patients studied. Furthermore, we demonstrate the utility of using bioreactors for the growth and survival of tumor cells. Our vision of being able to perform chemo-sensitivity tests in these bioreactors, in order to guide customized chemotherapeutic drug selection on a patient's own tumor cells, is now possible with these preliminary results.

15. SUBJECT TERMS
Cancer stem cells, bioreactors, ovarian cancer

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INTRODUCTION

Ovarian cancer is a nonspecific term describing a variety of cancers that originate in the ovary. There are about 20 microscopically distinct types of ovarian cancers. 90% of these cancers arise from surface epithelial cells, approximately 10% are germ cell tumors and the remaining cancers arise from specialized stromal cells. Today, ovarian cancer is the fifth leading cause of death among women. Patients with advanced disease have a poor prognosis with very low 5 year survival rates.

Over the years, the concept that tumors and normal tissues share the same structural organization has led to the identification of so-called cancer stem cells in several tissues such as head and neck[4] breast[1], brain[2, 3]. Cancer stem cells are thought to be responsible for the aggressiveness of the disease, metastasis and recurrence[5]. The central hypothesis of this proposal is that ovarian cancer is at least partly driven by a subpopulation of cells able to self renew, give rise to the tumor heterogeneity and drive tumorigenicity, i.e. cancer stem cells. A few studies identified CD44, CD117 and CD133 as ovarian cancer stem cell markers[6-8].

Major challenges in cancer research include the lack of *in vitro* models able to provide long-term tumor cell cultures as well as culture systems capable of sustaining cell growth and permitting structure formation similar to that found in patient tissue. Animal models have been used extensively and successfully to propagate human tumors, but many complex cellular interaction and molecular studies require the absence of the confounding host responses. Alternatively, limited *in vitro* 3-dimensional systems have been tentatively used. However, most of the studies focused on cancer cell lines but not on tumor cells found in ascites.

Our approach combines stem cell biology, cancer biology and bioengineering.

1) We first show that we have a 2-dimensional culture system able to specifically expand a population of cancer cells capable of long-term growth and self-renewal.

2) We propose to transpose this culture system to a 3-dimensional culture system using a bioreactor adapted from the bioartificial liver[10]. These bioreactors were originally developed and optimized for liver culture and were in clinical transplantation as a bridge for patients awaiting liver transplant[11].

BODY

Expansion of cancer stem cells.

8 samples of patients diagnosed with ovarian cancer were collected from ascites (Table 1). Each sample was plated on feeder cells, and although we successfully cultured 7 out of the 8 samples, only 4 of these samples could be reliably expanded in long-term cultures. Morphological studies show that two out of the four cultures grow as a monolayer of epithelial cells (OVC#1 and OVC#3). However, cell size and aspect appear very different from one sample to another. Two samples (OVC#4 and OVC#6) shows morphogenic 3-dimensional structures indicating a more complex cellular organization (Figure 1).
The observed heterogeneity of the samples cannot be related to the primary origin of the cancer. OVC#3 and OVC#6 are both serous cancers with metastasis in the omentum. Serous tumors resemble the glandular epithelial lining the fallopian tube. OVC#4 is an endometrioid adenocarcinoma and may contain cells resembling the endometrium (Table 2). In the next paragraph, we will see that this morphologic heterogeneity is reflected by the phenotype.

Flow cytometric study shows the expression of primitive markers.

Cells were harvested before the flasks reached confluency and analyzed by flow cytometry for expression of a wide variety of cell surface markers. These markers have been described in the literature as being expressed on stem cells and cancer stem cells. The flow cytometric analysis shows great heterogeneity within the samples and between the different samples (Figure 2A). Ascites is characterized by an accumulation of fluid in the abdominal cavity and is frequently observed in the pathogenesis of ovarian cancer. It involves

widespread dissemination of malignant ovarian epithelium throughout the entire peritoneal cavity. Therefore, ascites are expected to contain multiple cell types including inflammatory cells and tumor cells that have lost some of their adhesive capacity.

A few markers, such as EpCAM and CD166, were found to be homogeneous on all the samples, suggesting an epithelial origin of the tumor cells expanded in culture. We also investigated the expression of CD44 and CD133, both of which are described in the literature as cancer stem cell markers. All samples show different and varied levels of expression for CD133 and CD44, making it difficult to identify them as commonly relevant cancer stem cell markers in our hands. CD117 expression could not be studied by flow cytometry due to the epitope's sensitivity to trypsin. However, its expression was assessed by immunohistochemistry and we found this marker homogenously expressed in two of the cultures tested (Figure 2C).

Expansion of ovarian cancer stem cells in bioreactors.

Our next goal was to develop a 3-dimensional culture system better able to permit the development of the tumor architecture observed in patients. To achieve that, we have inoculated the tumor cells in bioreactors. Because of the limited availability of bioreactors, only three experiments using three different samples were attempted. Unfortunately, one bioreactor failed due to contamination and the experiment has not yet been repeated. In these experiments, bioreactors were initiated with stromal cells a few days before the tumor cells were injected. Bioreactor cultures were maintained for 3 weeks. Glucose consumption and lactate production were monitored on a daily basis to assess cell metabolism, and indirectly, tumor cell growth.

Tumor cell proliferation was also indirectly assessed by measuring CEA and CA125 levels in the circulating medium. CEA and CA125, belonging to the CEA antigen family, are produced by the tumor cells and released in the blood stream. CEA serum level is a clinical screening test for colon cancer, but some types of ovarian cancer also express CEA. CA125, although used as an indicator in some established ovarian cancer cases, cannot be reliably used as a clinical tool. Indeed, some cancer subtypes do not express CA125, and its expression is not specific to ovarian cancer. Both bioreactors exhibited a steady increase of CEA levels indicating tumor cell proliferation (Figure 3A). In the bioreactor seeded with OVC#4, CEA levels quickly reached a maximum level and remained constant until the end of the experiment. CA125 levels were undetectable in the 2-dimensional culture. The CA125 level in the bioreactor seeded with OVC#6 could not be detected. The low level of CA125 may be due to a dilution of the protein in the circulating medium as protein concentrations lower than 8.7U/ml are below the assay sensitivity. Unfortunately, we have been unable to obtain the CA125 circulating level for either patient so we were unable to make any comparison.

The degree of proliferation of the tumor cells and the percentage of proliferating tumor cells was determined by BrdU incorporation. BrdU, an analog of thymidine, was injected into the recirculation medium for the last 48h of the experiment, at a final concentration of 10µM. BrdU is incorporated into actively dividing cells and is revealed by Peroxydase staining (Figure 3B). Very few, less than 1% BrdU+ cells, could be found in any of the bioreactors. However, in OVC#6, we noticed that the distribution of these cells is irregular with some areas actively dividing, whereas other areas show sparsely scattered positive cells. The distribution of the BrdU+ cells in OVC#4 is more homogeneous, but few cells seem to have incorporated the dye. This result indicates that the tumor cells divide slowly or may have stopped proliferating before we injected the BrdU. A concurrent result is the CEA curve, which has reached a plateau a few days after initiation of the bioreactor. These results, the CEA and CA125 levels and BrdU incorporation, reflect the difference in the growth kinetics of these two samples.

Morphology of the tissue expanded in the bioreactor closely resembles the tissue.

Fibers were taken and fixed in formalin for paraffin embedding, or fixed in glutaraldehyde before being processed for transmission electron microscopy (Figure 4).

H&E analysis of both bioreactors show a difference in cellular organization as well as different cell size. OVC#4 bioreactor cells are smaller and show the most organization with the formation of what resembles a glandular epithelium. In contrast, OVC#6 shows a mass of cells which doesn't seem to present any specific organization. This may resemble the tissue structure in omentum metastasis. However, ultrastructure analysis on both samples shows that epithelial columnar cells compose the sum total of the bioreactor culture. These cells are characterized by the presence of tight junctions and villi borders. Although the cellular organization of the

3

two bioreactors seems different, formation of a "lumen" could occasionally be found in both cultures (Figure 4, green arrowhead). This can be put into perspective by the formation of glandular structures characteristic of most differentiated ovarian cancers.

Expression of markers is similar to what is observed in the patient.

To assess the similarity of the bioreactor cultures to the primary tumor, we obtained and stained slides of the original patient tissue. The omentum was available and is shown for all three samples, whereas the endometrium tumor is shown only for one sample OVC#4 (Figure 5). Each tissue was stained with CD133, CD44. EpCAM, a marker specific for epithelial cells, was used in conjunction with the other markers to specifically detect the tumor cells. In a tissue that is not normally composed by epithelial cells, large areas are EpCAM+ cells indicate the tumor cells. However, very few CD133+ cells are detected in OVC#4 whereas EpCAM and CD44 positive cells seem to co-localize. However, in the other samples, very few or no CD133 positive cells were found in the sections we looked at, and expression of CD44 and EpCAM seems to be mutually exclusive. It is of note that the CD133 expression pattern in tissues is always particular, as it is never homogeneously expressed at the cell surface. It is rather condensed at the apical border of the epithelial cells, as it was shed off the cell surface. This staining pattern has also been observed in colon adenocarcinoma (data not shown).

In our hands, these results do not seem to indicate that CD44 and EpCam are relevant stem cell markers for these tissues. However, we looked only at a portion of the tissue, which may not be representative of the entire tumor. For these reasons, we cannot be sure that the expression of these markers is not affected by the culture conditions in the bioreactors.

Phenotype of the cancer cells expanded in bioreactors is similar to those expanded in the 2-D culture system.

Fibers were collected and digested with collagenase, and the cell suspensions were stained with the same markers we used to characterize the 2-D cultured cells. The expression patterns of the different markers for the bioreactor cultures remained the same as the 2-D cultured cells, with the exception of a few adhesion markers (Figure 6). These few discrepancies can be explained by the fundamental differences between the two culture systems, i.e. the cell-to-cell interactions. Both CD44 and CD133 showed the same irregular expression in both samples. In addition, we did not observe a clear heterogeneity in the CD133 expression pattern. In one instance (OVC#6), due to difficulty in obtaining a sufficient quantity of cells, we re-cultured the cells isolated from the bioreactor by standard 2-D culture in order to reanalyze them after expansion. Previously, using a colon cancer tumor, we had shown that short-term re-culture in this manner does not significantly alter the tumor cell phenotype as the cells do not revert back to the 2-D phenotype (data not shown).

Cells expanded in bioreactors keep their tumorigenic potential.

To answer the question whether the cells we have expanded in bioreactor fulfill some of criteria of cancer stem cells i.e self renewal capacity and tumorigenicity, we have transplanted these cells into mice. One million tumor cells isolated from a bioreactor were injected sub-cutaneously into immunodeficient mice. About a month later, tumors were isolated and processed for flow cytometry and immunohistochemistry. Some of these isolated cells were serially re-injected into additional animals with the successful production of additional tumors. Results show the phenotype is conserved between the cultured cells and the xenografts. However, the expression of CD133 had changed and was now heterogeneous, as well as the CD44. Limiting dilution analysis done on these two CD133 subpopulations (CD133- and CD133+) showed a clear difference in their ability to form colonies. The ability of the cells to self-renew is maintained by the CD133+ population (data not shown), and correlates with an increased tumorigenic potential.

KEY RESEARCH ACCOMPLISHMENTS

This work has revealed the great heterogeneity characterizing the nature of ascites. Ovarian cancer is a complex disease and the variety of organs involved certainly reflects the nature of ascites. Although the

xenografts experiments confirm that the CD133 positive cells seem to possess a proliferation potential, we show that it is difficult to link any particular marker to the cancer stem cell hypothesis. A recent study suggests that CD133 positive cells contribute to the aggressiveness of the tumor by enhancing the tumor vascularization[12].

We have proof-of-concept that ovarian cancer cells from ascites can be grown and expanded in our 2-D culture system and moreover, that the heterogeneity of the sample is preserved throughout the entire culture process. We also show that it is possible to culture ovarian cancer cells in bioreactors and still preserve this heterogeneity. We believe these culture methods, both 2-D flask and 3-D bioreactor, will be of great importance in attaining future achievements towards the understanding of ovarian cancer pathology.

REPORTABLE OUTCOMES

Although these results are not definitive, we are writing a manuscript based on these results and other additional results. Better experimental models, permitting the improved understanding of the complexity and formation of ascites, need to be explored. Based on the findings reported here and the supposed orignin of the ascites samples we have, a future direction for this study could be seeding ovarian cancer cells in a fat-cell bioreactor in order to reconstitute the tumor as it occurs in the omentum.

A challenging idea in the field of ovarian cancer biology is the understanding of the various cell types composing ascites. We know that ascites arises from the dissemination of cancer cells through the abdominal wall into the peritoneal cavity, but determining the original source of these cells is essential to new breakthroughs in the field of cancer research. The availability of new mouse models would be of the greatest interest. The best method so far has been to inject the tumor cells subcutaneously in the neck region. But this method has limitations as it does not permit the study of the mechanism of tumor invasion or tumor formation in an organ.

CONCLUSION

We have shown that our *in vitro* culture system is suitable for the growth and expansion of a more primitive epithelial population of cancer cells, not only from solid tumors but also from ascites. Moreover, the culture system maintains the samples original heterogeneity. The hollow-fiber bioreactor is also a suitable tool for the study of tumor formation without interference from the microenvironment.

We have also shown that it is difficult to link any of the tested markers to the strict identification of ovarian cancer stem cells. We have shown on xenografts that cells expressing CD133 enrich for a population able to self-renew, confirming the findings of another study[8] but our results *in vitro* don't allow us to conclude with certainty that CD133 is a stem cell marker.

As our system selects preferentially for a more primitive epithelial population of cancer cells, it is possible to focus our studies on these tumor cells, regardless of the tumor's microenvironment.

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Sample patient history

Dr Deloia ID#	Date	Tumor/Patient	Ages (y.o)	Specimen	Race	Cancer type, stage, grade	metastasis status	Stage/Grade	Long-term Cultures	Tumors in mice?
TP06-252	3/7/06	OVA#1	45-50	ascites	Caucasian	clear cell carcinoma, primary (=ovary)	0/20	S3/G3	frozen	o out of 4
TP06-272	3/13/06	OVA#2	65-70	ascites	Caucasian	pap serous (ovary)	0/10	S3/G2	No	
TP06-691	6/8/06	OVA#3	45-50	ascites	UK	serous (peritoneal)	omentum	S3C/G3	P12	
TP06-799	6/30/06	OVA#4	55-60	ascites + tissue piece	Caucasian	endometroid (endometrium)	0/1	S4/G3	Contaminated (frozen)	
TP06-1107* / TP04-311CR	8/21/06	OVA#5	55-60	ascites	Caucasian	met adeno (ovary) recurrent	recurrent stage 2 colon negative	S4/G3	LOST (STROMA)	
TP06-1141 / SB06-135	8/25/06	OVA#6	50-55	ascites	Caucasian	met pap serous (peritoneal)	omentum, appendix, and peritoneum	S3C/G3	P7	1 out of 3
TP06-1706	11/28/06		75-80	ascites	Caucasian	Pap Serous (ovary)	omentum, peritoneum, and appendix	S3C/G3		
TP06-VG (no TP)	10/24/06	OVA#7	55- 60	ascites	Caucasian	pap serous (ovary)	recurrent refractory disease	S3C	P5	1 out of 3
TP07-238	2/7/07	OVA#8	50-55	ascites + tissue piece	Caucasian	clear cell carcinoma, (ovary)	omentum 0/20	S1A	LOST (STROMA)	

Table 1: Patient history of the 8 ascites samples received

Epithelial Ovarian cancer	
Serous	OVC#3 OVC#6 OVC#7
mucinous	
endometrioid	OVC#4
clear cell	OVC#1 OVC#8
papillary serous	
Brenner cell	
undifferentiated adenocarcinomas and sarcomas	OVC#5

Table 2: All ascites samples originate from epithelial ovarian cancers. This table represents the different samples grouped by origin.

Morphology of the cell cultures after *in vitro* expansion
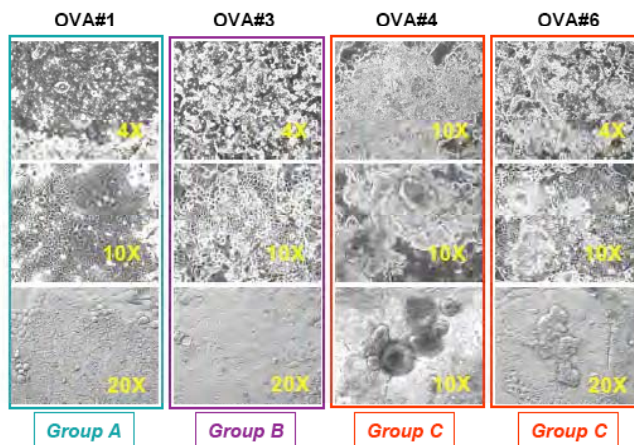

Figure 1: Ascites samples were plated on a feeder cell layer. 7 of 8 samples could be expanded *in vitro*, but only 4 samples were able to establish long-term cultures, with a stable morphology. OVC#1 and OVC#3 grow in a monolayer of epithelial cells. OVC#4 and OVC#6 displayed additionally 3-dimension like structures.
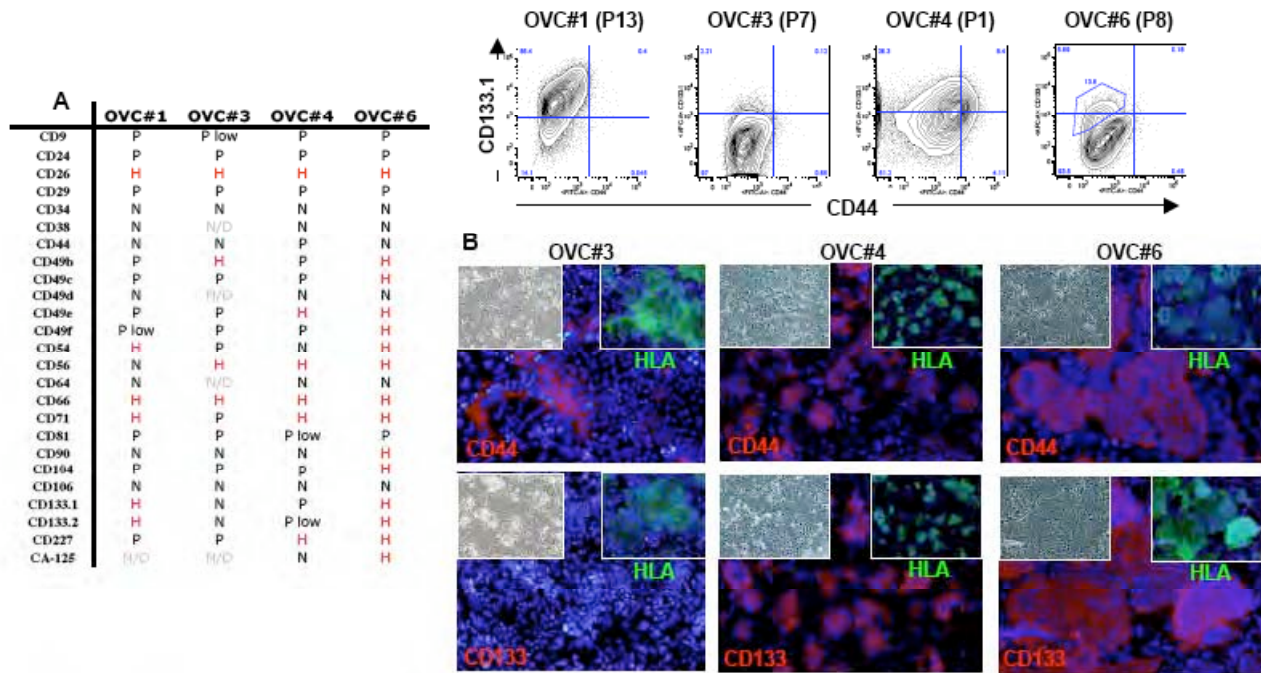
Phenotype of the cells expanded *in vitro*

A

	OVC#1	OVC#3	OVC#4	OVC#6
CD9	P	P low	P	P
CD24	P	P	P	P
CD26	H	H	H	H
CD29	P	P	P	P
CD34	N	N	N	N
CD38	N	N/D	N	N
CD44	N	N	P	N
CD49b	P	H	P	P
CD49c	P	P	P	H
CD49d	N	N/D	N	N
CD49e	P	P	H	H
CD49f	P low	P	P	H
CD54	H	P	N	H
CD56	N	H	H	H
CD64	N	N/D	N	N
CD66	H	H	H	H
CD71	H	P	H	H
CD81	P	P	P low	P
CD90	N	N	N	H
CD104	P	P	p	H
CD106	N	N	N	N
CD133.1	H	N	P	H
CD133.2	H	N	P low	H
CD227	P	P	H	H
CA-125	N/D	N/D	N	H

B

Figure 2: **A,** Before the flask reached confluency, tumor cells were harvested and stained with a set of monoclonal antibodies directed against molecules described as being expressed on stem cells and cancer stem cells. **B,** Cells were cultured on chamber slides until they reach about 70% confluency. Slides were stained with CD44 or polyclonal CD133 (red), co-stained with HLA-ABC (green) and counterstained with Hoechst (blue).
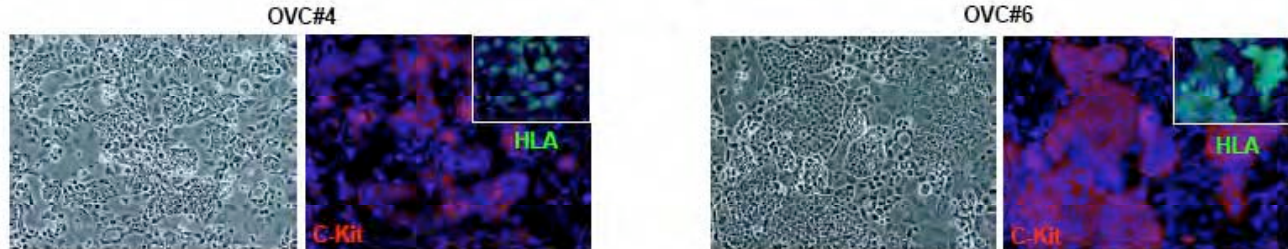
Figure 2: **C,** Cells were cultured on chamber slides until they reach about 70% confluency. Slides were stained with c-kit (red), co-stained with HLA-ABC (green) and counterstained with Hoechst (blue).
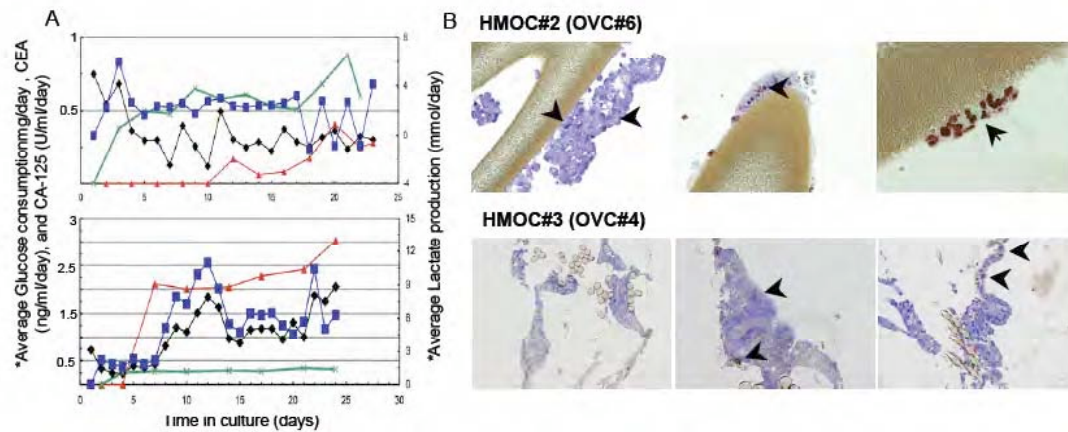
Bioreactor experiments: results

Figure 3: **A,** Circulating medium was collected each day and stored until further analyses were done. Lactate production (blue curve) and glucose consumption (black curve), CEA (red curve), and CA-125 (green curve) were measured to assess the global metabolic functions of the cells. CEA was used to specifically measure the activity of the tumor cells in the bioreactor. **B,** Fibers was fixed in formallin for paraffin embedding. Slides were stained with an anti-BrdU antibody and revealed by Peroxydase reaction (brown staining; black arrowheads). Hematoxylin counterstain visualizes the nuclei.

* All values are expressed per 10^6 inoculated cells)
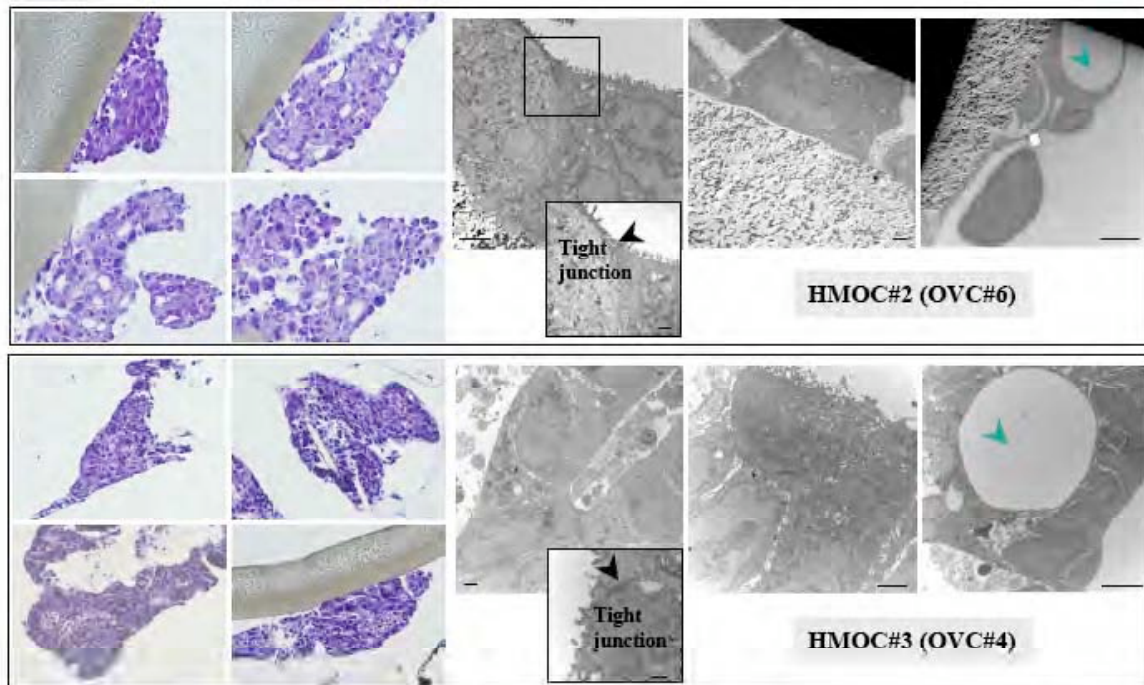
Bioreactor: morphology

Figure 4: Fibers from both experiments were collected and paraffin embedded (Top panel: OVC#6; Bottom panel: OVC#4). H&E staining and electron microscopy were performed to assess the general morphology of the cells. Morphologic analysis shows a mass of cells with different levels of organization. Ultrastructure analysis of both experiments usually shows a single layer of epithelial columnar cells, characterized by the presence of villi border and tight junctions. The possible formation of a glandular structure can also be seen (green arrowhead).
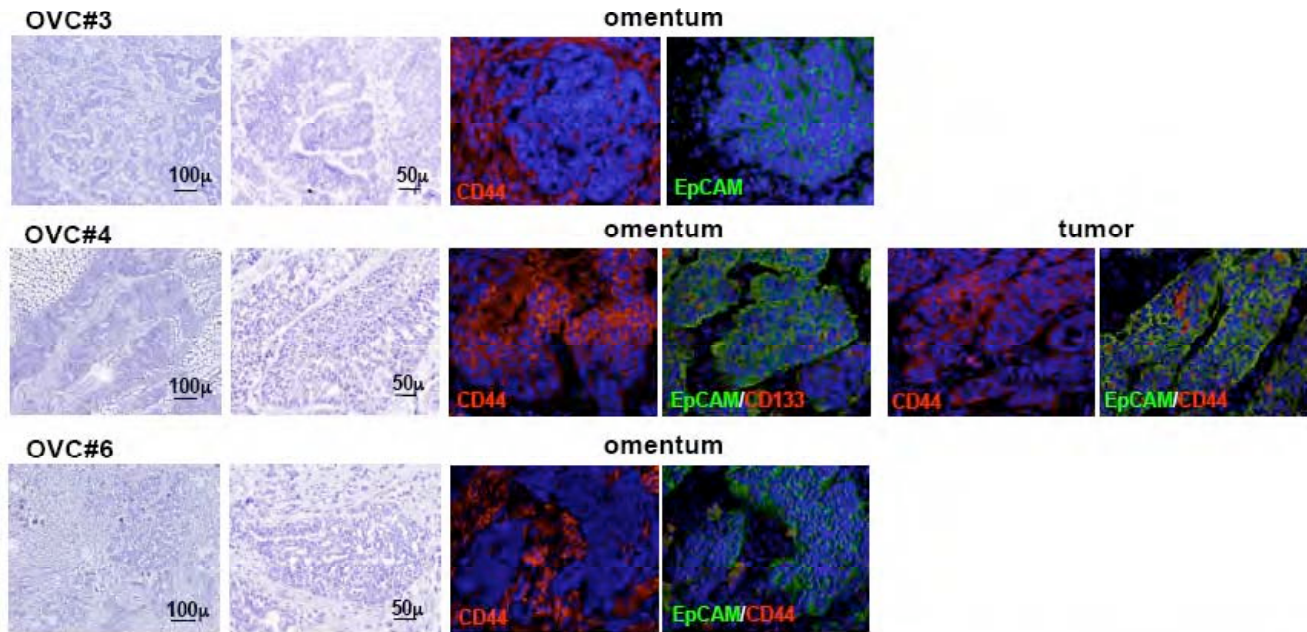
Histology of the tumor in the patient

Figure 5: H&E and immunohistochemistry histology of the solid tumor in the omentum is represented here. The omentum is composed of islets of epithelial tumor cells among disorganized areas composed with stroma, matrix. An EpCAM staining identifies the nests of epithelial tumor cells. However, whether these cells are CD44 and CD133 differ between the samples. Only in OVC#4 these cells express CD44, whereas some are CD133. In OVC#3 and OVC#6, the CD44+ cells are EpCAM-.
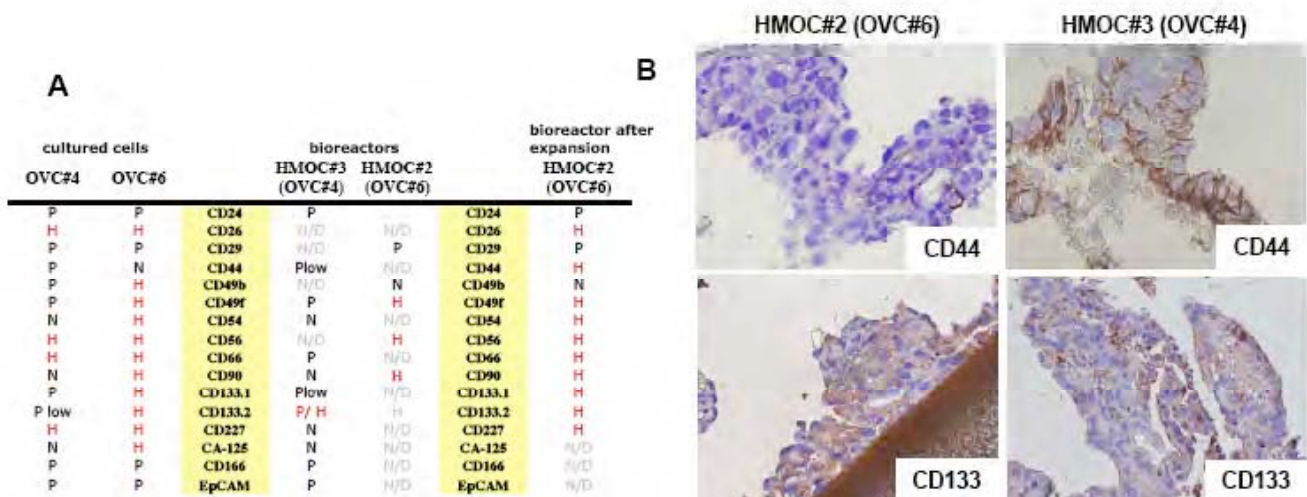
Bioreactors: structure and phenotype

A

cultured cells			bioreactors			bioreactor after expansion
OVC#4	OVC#6		HMOC#3 (OVC#4)	HMOC#2 (OVC#6)		HMOC#2 (OVC#6)
P	P	CD24	P		CD24	P
H	H	CD26	N/D	N/D	CD26	H
P	P	CD29	N/D	P	CD29	P
P	N	CD44	Plow	N/D	CD44	H
P	H	CD49b	N/D	N	CD49b	N
P	H	CD49f	P	H	CD49f	H
N	H	CD54	N	N/D	CD54	H
H	H	CD56	N/D	H	CD56	H
H	H	CD66	P	N/D	CD66	H
N	H	CD90	N	H	CD90	H
P	H	CD133.1	Plow		CD133.1	H
P low	H	CD133.2	P/ H	H	CD133.2	H
H	H	CD227	N	N/D	CD227	H
N	H	CA-125	N	N/D	CA-125	N/D
P	P	CD166	P	N/D	CD166	N/D
P	P	EpCAM	P	N/D	EpCAM	N/D

B

Figure 6: **A,** A portion of the fibers were collected and digested with collagenase. The cell suspension was stained according to the flow cytometry standard procedure with the same markers that were used on the 2-D cultured cells. Due to the difficulty in obtaining a sufficient amount of cells, the cell suspension was then cultured and analyzed after expansion. **B,** Fibers were also stained with a variety of markers, including CD44 and CD133, and revealed by peroxydase (HRP) reaction.
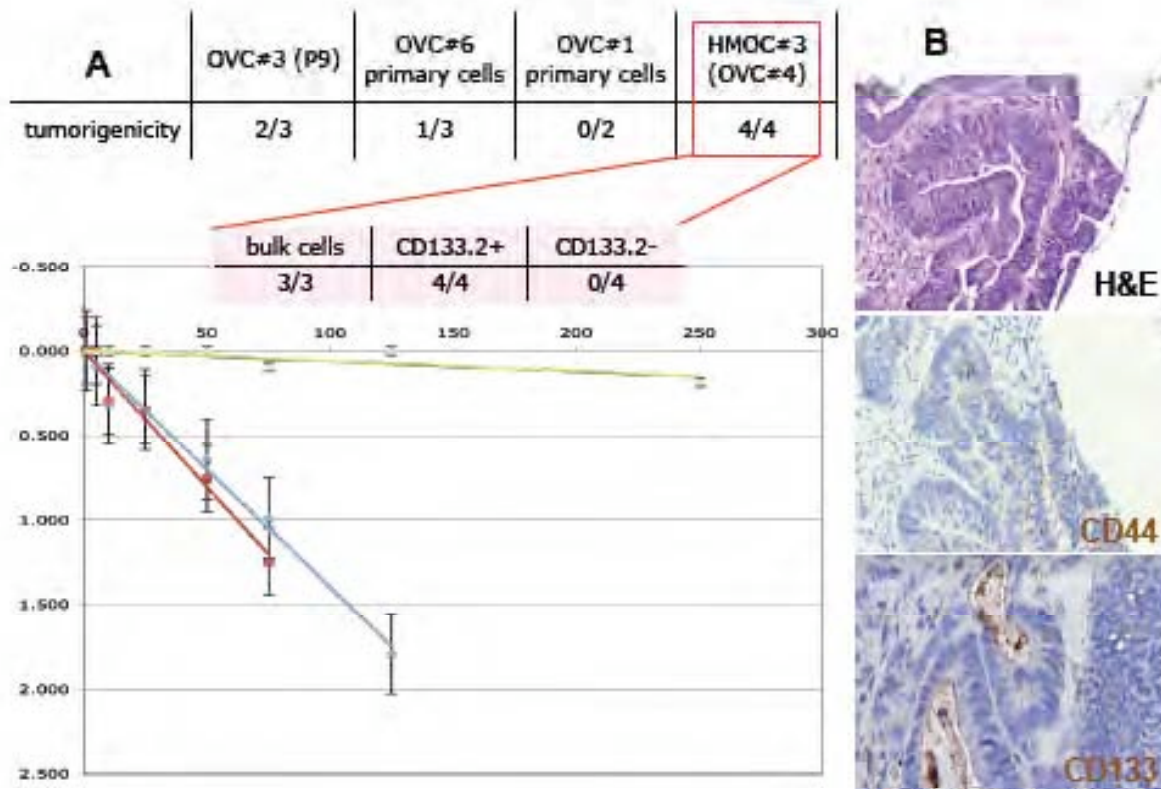
10

Transplantation data

Figure 7: A, Cells isolated from the bioreactor HMOC#3 seeded with OVC#4 were injected subcutaneously into mice. Tumors were successfully generated and CD133.2⁺ cells show an increased proliferation capacity that correlates with the ability to induce a tumor. **B**, Xenografts were stained with CD44 and CD133 expression. Both markers are heterogeneously expressed.